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ANTIGEN COMPOSITION AGAINST MYCOPLASMA

The present invention relates to protective and diagnostic antigens, the preparation thereof, and their use in the formation of vaccine compositions, particularly vaccine compositions against Mycoplasma hyopneumoniae infections.

Mycoplasma hyopneumoniae is a ubiquitous swine respiratory pathogen causing mycoplasmal pneumoniae in swine (swine enzootic pneumonia). Swine enzootic pneumonia is probably the most widespread and economically significant disease in swine producing countries of the world. The economic effects of swine enzootic pneumonia (SEP) are complex, and the cost of the disease is severe. In Australia, the disease was estimated in 1988 to cost approximately $20,000,000 per annum. Increased mortality, decreased growth weight, depressed feed conversion, susceptibility to secondary bacterial infections, increased management costs, and increased use of antibiotics, are the main reasons for the economic impact of SEP.

Whilst several experimental vaccines have been produced, these have resulted in less than optimal results, and utilising various classes of antibiotics such as tetracycline, lincamycin and tiamulin is still the most widespread control treatment. Such antibiotics are, however, of limited therapeutic value, because they do not prevent the establishment of an infection, and lung lesions may develop after treatment ends.

European Patent Application 359,919 to ML Technology Ventures L.P. describes a series of antigens, 36 kD, 41 kD, 74.5 kD and 96 kD in size, and proposes the use of such antigens in vaccines. Results presented suggest that some protection in pigs against challenge was achieved.

However, there remains a need in the art for an effective vaccine against M. hyopneumoniae which would confer protection against colonisation and clinical disease following M. hyopneumoniae challenge and also significantly reduce the morbidity and mortality from secondary infections

Accordingly, it is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties and deficiencies in the prior art.

Accordingly, in a first aspect of the present invention there is provided a putative protective antigen against a Mycoplasma, preferably Mycoplasma

hyopneumoniae prepared by a method including

providing

a sample of a Mycoplasma;

an antibody probe including at least one antibody against a Mycoplasma produced by a method including;

providing a biological sample taken a short time after an immune animal has been challenged with a Mycoplasma or Mycoplasma extract taken from the infection site or an area of a lesion or an area close to the infection site or lesion;

isolating cells from the biological sample;

culturing cells in vitro in a suitable culture medium; and

harvesting antibodies produced from said cells;

probing the Mycoplasma sample with the antibody probe to detect at least one antigen; and

isolating the antigen detected.

The protective antigens may also function as diagnostic antigens as discussed below.

Accordingly, in a preferred aspect of the present invention there is provided a putative protective antigen against Mycoplasma hyopneumoniae, or related infections, selected from the group of antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kilodaltons (kD), as hereinafter described, mutants, derivatives and fragments thereof. The putative protective antigen may be a surface protein. The putative protective antigen may be a surface lipoprotein or membrane protein.

Preferably the protective antigens are selected from the group of antigens having approximate molecular weights of 110-114, 90-94, 74, 62, 52 and 48 kD.

B Preferably, the 72-75 kD antigen includes the following N-terminal amino acid sequence (SEQ ID NO:12):

AGXLQKNSLLEEVWYLAL

B and, optionally, one or more of the following internal amino acid sequences (SEQ ID NO:13; SEQ ID NO:14; and SEQ ID NO:15 respectively):

AKNFDFAPSIQGYKKIAHEL

NLKPEQILQLLG

LLKAEXNKXIEEINTXLDN

Preferably, the 60-64 kD antigen includes one of the following N-terminal amino acid sequences (SEQ ID NO: 10 and SEQ ID NO: 11 respectively):

MKLAKLLKGFX(N/L)(M/V)IK

ADP(F/I)(R/E)Y(V/A)PQG(Q/A)X(M/N)VG

Preferably, the 52-54 kD antigen includes the following N-terminal amino acid sequence (SEQ ID NO: 7):

AGXWAKETTKEEKS

and, optionally, one or more of the following internal amino acid sequences (SEQ ID NO: 8 and SEQ ID NO: 9 respectively):

AWVTADGTVN

AIVTADGTVNDNKPNQWVRKY.

Preferably, the 46-48 kD antigen includes the following N-terminal amino acid sequence (SEQ ID NO: 3):

- AGXGQTESGSTSDSKPQAETLKHKV

and, optionally, one or more of the following internal amino acid sequences (SEQ ID NO: 4; SEQ ID NO: 5; and SEQ ID NO: 6 respectively):

TIYKPDKVLGKVAVEVLRVLIAKKNKASR

AEQAITKLKLEGFDTQ

KNSQNKIIDLSPEG

The 46-48 kD antigen may be encoded by a nucleic acid fragment (SEQ ID NO: 1):

10 1234567890	20 1234567890	30 1234567890	40 1234567890	50 1234567890	
ATGAAAAAAA	TGCCACTATA	CCAGAGGAAA	GAGCAGTATA	TAAAATAATT	50
AAAATTACAT	TTTCTTCATT	TGCGCCAGAA	TTTTTAAGAA	TTAGTACATT	100
AAAAAGTAGA	ACAAAAGTTA	TTAATGTAAA	CATTAGCGCA	ATCCTTAAGA	150
AAAAATTAAA	AGTTTTATCT	ATTTTTTTTA	ATCGAAATCC	AACCAGGCAT	200
AAATCTTTGT	CAGTATTTAT	CAAGTCGGTA	TTTTTTCATT	ATTTCTACTA	250
AAATATTATT	TGAATTTGCA	TTTTCCATAA	TCTAAAATTT	TACATTTTTT	300
TATAACAATT	TTTAAAAATT	ACTCTTTAAT	TTATAGTATT	TTTTTATTTT	350
TTAGTCTAAA	TTATAAAATT	ATCTTGAATT	TTATTTGAAT	TTTTATAATT	400
TAGTACTAAA	AAATACAAAT	ATTTTTTCCT	ATTCTAAGAA	AAATTCATTT	450
TTTAAAAAAA	ATTGATTTTT	ATAGTATAAT	TTGTTTGTAT	AATTGAATTA	500
ACTTGATTTG	AAAGGGAACA	AAATGAAAAA	AATGCTTAGA	AAAAAATTCT	550
TGTATTCATC	AGCTATTTAT	GCAACTTCGC	TTGCATCAAT	TATTGCATTT	600
GTTGCAGCAG	GTTGTGGACA	GACAGAATCA	GGTTCAACTT	CTGATTCTAA	650
ACCACAAGCC	GAGACGCTAA	AACATAAAGT	AAGTAATGAT	TCTATTCGAA	700
TAGCACTAAC	CGATCCGGAT	AATCCTCGAT	GAATTAGTGC	CCAAAAAGAT	750
ATTATTTCTT	ATGTTGATGA	AACAGAGGCA	GCAACTTCAA	CAATTACAAA	800
AAACCAGGAT	GCACAAAATA	ACTGACTCAC	TCAGCAAGCT	AATTTAAGCC	850
CAGCGCCAAA	AGGATTTATT	ATTGCCCCTG	AAAATGGAAG	TGGAGTTGGA	900

ACTGCTGTTA	ATACAATTGC	TGATAAAGGA	ATTCCGATTG	TTGCCTATGA	950
TCGACTAATT	ACTGGATCTG	ATAAATATGA	TTGGTATGTT	TCTTTTGATA	1000
ATGAAAAAGT	TGGTGAATTA	CAAGGTCTTT	CACTTGCTGC	GGGTCTATTA	1050
GGAAAAGAAG	ATGGTGCTTT	TGATTCAATT	GATCAAATGA	ATGAATATCT	1100
AAAATCACAT	ATGCCCCAAG	AGACAATTTC	TTTTTATACA	ATCGCGGGTT	1150
CCCAAGATGA	TAATAATTCC	CAATATTTTT	ATAATGGTGC	AATGAAAGTA	1200
CTTAAAGAAT	TAATGAAAAA	TTCGCAAAAT	AAAATAATTG	ATTTATCTCC	1250
TGAAGGCGAA	AATGCTGTTT	ATGTCCCAGG	ATGAAATTAT	GGAACTGCCG	1300
GTCAAAGAAT	CCAATCTTTT	CTAACAATTA	ACAAAGATCC	AGCAGGTGGT	1350
AATAAAATCA	AAGCTGTTGG	TTCAAAACCA	GCTTCTATTT	TCAAAGGATT	1400
TCTTGCCCCA	AATGATGGAA	TGGCCGAACA	AGCAATCACC	AAATTAAAAC	1450
TTGAAGGGTT	TGATACCCAA	AAAATCTTTG	TAACTCGTCA	AGATTATAAT	1500
GATAAAGCCA	AAACTTTTAT	CAAAGACGGC	GATCAAAATA	TGACAATTTA	1550
TAAACCTGAT	AAAGTTTTAG	GAAAAGTTGC	TGTTGAAGTT	CTTCGGGTTT	1600
TAATTGCAAA	GAAAAATAAA	GCATCTAGAT	CAGAAGTCGA	AAACGAACTA	1650
AAAGCAAAAC	TACCAAATAT	TTCATTTAAA	TATGATAATC	AAACATATAA	1700
AGTACAAGGT	AAAAATATTA	ATACAATTTT	AGTAAGTCCA	GTAATTGTTA	1750
CAAAAGCTAA	TGTTGATAAT	CCTGATGCCT	AA		1782

B Accordingly, in a further aspect the present invention provides an isolated nucleic acid fragment encoding a putative protective antigen against Mycoplasma hyopneumoniae or related infections, said nucleic acid fragment (SEQ ID NO:1):

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
ATGAAAAAAA	TGCCACTATA	CCAGAGGAAA	GAGCAGTATA	TAAAATAATT	50
AAAATTACAT	TTTCTTCATT	TGCGCCAGAA	TTTTTAAGAA	TTAGTACATT	100
AAAAAGTAGA	ACAAAAGTTA	TTAATGTAAA	CATTAGCGCA	ATCCTTAAGA	150
AAAAATTAAA	AGTTTTATCT	ATTTTTTTTA	ATCGAAATCC	AACCAGGCAT	200
AAATCTTTGT	CAGTATTTAT	CAAGTCGGTA	TTTTTTCATT	ATTTCTACTA	250
AAATATTATT	TGAATTTGCA	TTTTCCATAA	TCTAAAATTT	TACATTTTTT	300
TATAACAATT	TTTAAAAATT	ACTCTTTAAT	TTATAGTATT	TTTTTATTTT	350
TTAGTCTAAA	TTATAAAATT	ATCTTGAATT	TTATTTGAAT	TTTTATAATT	400
TAGTACTAAA	AAATACAAAT	ATTTTTTCCT	ATTCTAAGAA	AAATTCATTT	450
TTTAAAAAAA	ATTGATTTTT	ATAGTATAAT	TTGTTTGTAT	AATTGAATTA	500
ACTTGATTTG	AAAGGGAACA	AAATGAAAAA	AATGCTTAGA	AAAAAATTCT	550
TGTATTCATC	AGCTATTTAT	GCAACTTCGC	TTGCATCAAT	TATTGCATTT	600
GTTGCAGCAG	GTTGTGGACA	GACAGAATCA	GGTTCAACTT	CTGATTCTAA	650
ACCACAAGCC	GAGACGCTAA	AACATAAAGT	AAGTAATGAT	TCTATTCGAA	700
TAGCACTAAC	CGATCCGGAT	AATCCTCGAT	GAATTAGTGC	CCAAAAAGAT	750
ATTATTTCTT	ATGTTGATGA	AACAGAGGCA	GCAACTTCAA	CAATTACAAA	800
AAACCAGGAT	GCACAAAATA	ACTGACTCAC	TCAGCAAGCT	AATTTAAGCC	850
CAGCGCCAAA	AGGATTTATT	ATTGCCCCTG	AAAATGGAAG	TGGAGTTGGA	900
ACTGCTGTTA	ATACAATTGC	TGATAAAGGA	ATTCCGATTG	TTGCCTATGA	950
TCGACTAATT	ACTGGATCTG	ATAAATATGA	TTGGTATGTT	TCTTTTGATA	1000
ATGAAAAAGT	TGGTGAATTA	CAAGGTCTTT	CACTTGCTGC	GGGTCTATTA	1050
GGAAAAGAAG	ATGGTGCTTT	TGATTCAATT	GATCAAATGA	ATGAATATCT	1100
AAAATCACAT	ATGCCCCAAG	AGACAATTTC	TTTTTATACA	ATCGCGGGTT	1150
CCCAAGATGA	TAATAATTCC	CAATATTTTT	ATAATGGTGC	AATGAAAGTA	1200
CTTAAAGAAT	TAATGAAAAA	TTCGCAAAAT	AAAATAATTG	ATTTATCTCC	1250
TGAAGGCGAA	AATGCTGTTT	ATGTCCCAGG	ATGAAATTAT	GGAACTGCCG	1300
GTCAAAGAAT	CCAATCTTTT	CTAACAATTA	ACAAAGATCC	AGCAGGTGGT	1350

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AATAAAATCA AAGCTGTTGG TTCAAAACCA GCTTCTATTT TCAAAGGATT 1400
TCTTGCCCCA AATGATGGAA TGGCCGAACA AGCAATCACC AAATTAAAAC 1450
TTGAAGGGTT TGATACCCAA AAAATCTTTG TAACTCGTCA AGATTATAAT 1500
GATAAAGCCA AAACTTTTAT CAAAGACGGC GATCAAAATA TGACAATTTA 1550
TAAACCTGAT AAAGTTTTAG GAAAAGTTGC TGTTGAAGTT CTTCGGGTTT 1600
TAATTGCAAA GAAAAATAAA GCATCTAGAT CAGAAGTCGA AAACGAACTA 1650
AAAGCAAAAC TACCAAATAT TTCATTTAAA TATGATAATC AAACATATAA 1700
AGTACAAGGT AAAAATATTA ATACAATTTT AGTAAGTCCA GTAATTGTTA 1750
CAAAAGCTAA TGTTGATAAT CCTGATGCCT AA                    1782
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As cross protection between various Mycoplasma such as M. hyorhinis and M. synoviae has been documented, similar antigens may also be detected in other Mycoplasma species (Figure 1).

In a still further aspect the present invention provides a method for preventing Mycoplasma infection in animals. Preferably the Mycoplasma disease is a Mycoplasma hyopneumoniae disease such as swine enzootic pneumonia (SEP). This method includes administering to an animal an effective amount of at least one protective antigen against Mycoplasma as described above.

The present invention further provides a vaccine composition including a prophylactically effective amount of at least one putative protective antigen against a Mycoplasma as herein described. Preferably the veterinary composition includes two or more putative protective antigens as herein described.

Accordingly in a preferred aspect the present invention provides a vaccine composition including two or more putative protective antigens selected from antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kilodaltons.

The vaccine composition may include any combination of two or more putative protective antigens selected from antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kD. The two or more antigens may be selected from antigens falling within one of the specified approximate molecular weights and/or antigens from different specified approximate molecular weights. The composition may contain 3, 4, 5 or 6 antigens selected from protective antigens having molecular weights of approximately 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kD.

The vaccine compositions according to the present invention may be

administered orally or may be administered parenterally (for example by intramuscular, subcutaneous, intradermal or intravenous injection). The amount required will vary with the antigenicity of the active ingredient and need only be an amount sufficient to induce an immune response typical of existing vaccines.

Reactive experimentation will easily establish the required amount. Typical initial doses of vaccine or veterinary compositions may be approximately 0.001-1 mg active ingredient/kg body weight. The dose rate may increase or multiple doses may be used as needed to provide the desired level of protection.

The vaccine composition according to the present invention may further include a veterinary acceptable carrier, diluent or excipient therefor. Preferably the active ingredient may be suspended or dissolved in a carrier. The carrier may be any solid or solvent that is nontoxic to the animal and compatible with the active ingredient. Suitable carriers include liquid carriers, such as normal saline and other nontoxic salts at or near phsyiological concentrations, and solid carriers, such as talc or sucrose.

Preferably the vaccine contains an adjuvant, such as Freund's adjuvant, complete or incomplete, or immunomodulators such as cytokines may be added to enhance the antigenicity of the antigen if desired.

More preferably the adjuvant is of the mineral-oil type as these have been found to be consistently superior at inducing antibody titres and Delayed Type Hypersensitivity responses. A particularly preferred adjuvant is that marketed under the trade designation Montanide ISA-50 and available from Seppic, Paris, France.

When used for administering via the bronchial tubes, the vaccine is suitably present in the form of an aerosol.

In a still further aspect of the present invention there is provided a diagnostic kit including a diagnostic antigen against a Mycoplasma, preferably Mycoplasma hyopneumoniae, identified and purified as described above.

The putative protective antigens according to the present invention may be isolated and identified utilising the general methods described in Australian patent application 49035/90, the entire disclosure of which is incorporated herein by reference

Accordingly, in a further aspect, the present invention provides a method for producing at least one antibody against a Mycoplasma. This method includes

providing a biological sample taken a short time after an immune animal has been challenged with a Mycoplasma or Mycoplasma extract taken from the infection site or an area of a lesion or an area close to the infection site or lesion;

isolating cells from the biological sample;

culturing cells in vitro in a suitable culture medium; and

harvesting antibodies produced from said cells.

The Mycoplasma may be Mycoplasma hyopneumoniae.

The animal may be a mammal including humans. The mammal may be a domestic animal such as a pig, sheep or cattle.

The biological animal sample may be of any suitable type. The biological sample may be taken from animal tissue, organs, lymph or lymph nodes. The biological sample may be taken from the infection site, the lungs of the animal, or an area of a lesion which may be formed or an area close to the infected site or a lesion such as in the lymph nodes draining from the lungs.

However, serum/plasma samples are not used as the biological samples according to this aspect of the present invention. It has been found that the majority of antibodies found in a serum/plasma sample are irrelevant to protection or specific diagnosis or a Mycoplasma or are unrelated to the Mycoplasma. In addition, other serum/ plasma components may interfere with the specific reactions between pathogen components and antibodies to them.

In contrast, the probes described in the present invention are highly enriched in Mycoplasma-specific antibodies of particular importance to protective immunity.

It is preferred that the biological samples are taken from the animals at a predetermined time in the development of the disease. In general, for a Mycoplasma infection, it has been found that the biological samples should be taken approximately 2 to 7 days after challenge with or after administration of products obtained from a pathogen or with the pathogen itself.

The cells isolated from the biological sample may include B cells.

Thus, preferably the cells are taken a short time after in vivo stimulation.

preferably within approximately 2 to 5 days thereafter, resulting in the in vivo induction of antibody forming cells which will secrete specific antibodies into the culture medium after in vitro incubation.

In vitro secretion of antibodies in the culture medium by recently activated B cells may be enhanced by the addition of helper factors to the cultures. The helper factors may be cytokines used alone or in combination, including Interleukin 1, 2, 3, 4, 5, 6, 7 and 8, colony stimulating factors, interferons and any other factors that may be shown to have an enhancing effect on specific B cell secretion.

The method of producing an antibody may include a further step of activating the cells isolated to proliferate and secrete and/or release antibodies.

The cell activation step may include adding a cell activating agent to the culture medium. The cell activating agent may be selected from mitogens and helper factors produced by leukocytes, or their synthetic equivalents or combinations thereof.

The mitogens may be selected from the group including products derived from pokeweed (Phytolacca americana) also known as pokeweed mitogen (PWM), polyvinylpyrrolidone (PVP), polyadenylic-polyuridylic acid (poly(A-U)), purified protein derivate (PPD), polyinosinic-polycytidilic acid (poly(I-C)), lipopolysaccharide (LPS), staphylococcal organisms or products thereof, Bacto-streptolysin O reagent (SLO), Staphylococcal phage lysate (SPL), Epstein-Barr virus (EBV), Nocardia water-soluble mitogen (NWSM), phytohemagglutinin (PHA), Concanavalin A (Con A), and dextran-sulphate and mixtures thereof. The cell proliferation agent may be any agent that indirectly or directly results in B cell proliferation and/or antibody secretion such as solid-phase anti-immunoglobulin. The helper factors may be selected from the group including cytokines including interleukin 1, 2, 3, 4, 5, 6, 7 and 8, colony stimulating factors, interferons and any other helper factors that may be shown when added alone, or in combination with other factors and agents, to have an enhancing effect on specific B cell proliferation and/or antibody secretion. This in no way is meant to be an exhaustive list of mitogens and cell actuating agents including helper factors.

The in vitro culturing of the cells may be conducted with or without prior

steps to separate sub-populations of cells. The harvesting of antibodies may be conducted by harvesting of the supernatant from the culture medium. This supernatant contains antibodies secreted by these cells during the *in vitro* culture or artificially released from the B cells, for example by lysis of the B cells. It has been found that the antibody-containing supernatants may be used directly to detect antigens of the *Mycoplasma*.

In a preferred aspect of the present invention, there is provided a method for identifying an antigen associated with a *Mycoplasma*, preferably *Mycoplasma hyopneumoniae*. This method includes

providing

a sample of a *Mycoplasma*; and

an antibody probe including at least one antibody against a *Mycoplasma*;

probing the *Mycoplasma* sample with the antibody probe to detect at least one antigen; and

isolating the antigen detected.

The sample of *Mycoplasma* may be mixed with a standard buffer solution and placed on a standard support such as an SDS-polyacrylamide gel to separate the proteins contained thereon (Figure 2).

Alternatively, the proteins may be selected utilising the non-ionic detergent Triton X-114 (TX-114). Insoluble material may be removed by centrifugation. Proteins soluble in the TX-114 phase may then be precipitated out (Figure 2).

The separate proteins may then be transferred to nitrocellulose, nylon or other sheets.

The probing with a suitable antibody may further include subjecting the product produced thereby to a detection assay. The detection assay may include Western blot techniques. The detection assay may be an immunoprecipitation assay, a radioimmunoassay, an enzyme-linked immunoassay or immunofluorescent assay (Figures 3, 4 and 5)

The antibody produced as described above may be utilized simply in the form of the supernatant harvested from the culture medium. Alternatively, the antibodies may be separated and purified

In a further preferred aspect of the present invention the antibody contained in the culture medium may be used for the affinity purification, preferably immuno-affinity purification of antigen.

Accordingly, in a preferred aspect there is provided a method for purifying antigen. This method includes

providing

a crude antigen mixture; and

an antibody against a Mycoplasma immobilized on a suitable support;

subjecting the crude antigen mixture to affinity chromatography utilizing the immobilized antibody; and

isolating the purified antigen so formed.

The antibody is produced by the method described above.

Antibody can be obtained from the culture supernatant probe by conventional methods. For example, methods usually used to purify immunoglobulins from serum or plasma, e.g. precipitation with ammonium sulphate, fractionation with caprylic acid, ion exchange chromatography, or by binding and elution from immobilized protein G or protein A, may be utilized. Antibody so obtained can then be coupled to suitable supports, e.g., CNBr-activated Sepharose 4B (Pharmacia), Affi-gel (Bio-RAD), or other affinity chromatography supports able to bind proteins.

Immobilized antibody can then be applied to the fractionation and purification of specific antigen from a complex Mycoplasma extract by affinity chromatography. After binding of antigen to immobilized antibody, unbound macromolecular species can be washed away from the solid support with, e.g. buffers containing 1.5 M NaCl. Subsequently the antigen can be eluted from the affinity column with, e.g. low or high pH buffer or buffers containing chaotropic ions, e.g. 0.5-3.0 M sodium thiocyanate.

The application of the antibody probe to affinity chromatography enables sufficient quantities of specific antigens to be rapidly isolated from a complex crude extraction mixture for biochemical characterization, amino-acid sequencing and vaccination of animal for limited protection studies. Application of affinity

chromatography for obtaining antigen(s) avoids the difficulties often encountered when applying conventional biochemical techniques to the purification of an antigen about which little or no data is known. It also obviates the need to raise polyclonal or monoclonal antibodies for the purpose of "analytical" affinity chromatography. Large scale preparation may, however, require the preparation of polyclonal or monoclonal antibodies.

Having identified the antigen(s) molecular biology, chemical techniques, e.g. cloning techniques, may be used to produce unlimited amounts of this antigen or, alternatively, synthetic peptides corresponding to different fragments of the identified antigens may be used as a means to produce a vaccine.

Accordingly in a preferred aspect of the present invention there is provided a method for preparing a synthetic antigenic polypeptide against Mycoplasma, preferably Mycoplasma hyopneumoniae, which method includes

providing

a cDNA library or genomic library derived from a sample of Mycoplasma; and

an antibody probe as described above;

generating synthetic polypeptides from the cDNA library or genomic library;

probing the synthetic polypeptides with the antibody probe; and

isolating the synthetic antigenic polypeptide detected thereby.

Either cDNA or genomic libraries may be used. The cDNA or genomic libraries may be assembled into suitable expression vectors that will enable transcription and the subsequent expression of the clone cDNA, either in prokaryotic hosts (e.g. bacteria) or eukaryotic hosts (e.g. mammalian cells). The probes may preferably be selected from

(i) synthetic oligonucleotide probes based on the amino acid sequence of the antigen identified and purified as described above;

(ii) antibodies obtained from the culture medium produced as described above;

(iii) monoclonal or polyclonal antibodies produced against the antigens identified and purified as described above;

(iv) recombinant or synthetic monoclonal antibodies or polypeptides with

specificity for the antigen, e.g. as described by Ward et al., Nature, 241, pages 544-546 (1989).

The synthetic antigenic polypeptide produced in accordance with the invention may be a fusion protein containing the synthetic antigenic peptide and another protein.

In a further aspect of the present invention there is provided a DNA fragment encoding a putative protective antigen against Mycoplasma or related infections, said DNA fragments having a nucleic acid sequence according to Figure 6a and 6b or an homologous sequence and functionally active fragments thereof.

In a further preferred aspect of the present invention there is provided a clone including a DNA fragment encoding a putative protective antigen against Mycoplasma or related infections, said DNA fragments having a nucleic acid sequence according to Figure 6a and 6b or an homologous sequence and functionally active fragments thereof.

Preferably the clone is pC1-2.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

IN THE FIGURES:

FIGURE 1: SDS-Polyacrylamide gel (12.5%) profiles of SDS extracts of species of mycoplasma- Coomassie R250 stained.

Lane 1 Pre-stained Molecular Weight Standards

Lane 2 *M. gallisepticum*

Lane 3 *M. synoviae*.

Lane 4 *M. hyopneumoniae*.

Lane 5 *M. hyorhinis*.

Lane 6 *M. flocculare*.

FIGURE 2: SDS-Polyacrylamide gel (12.5%) profiles of extracts of strains of *M. hyopneumoniae* - Coomassie R250 stained gel

Lane 1 Pre-stained Molecular Weight Standards.

Lane 2 Triton X-114 extract of *M. hyopneumoniae* - strain Beaufort.

Lane 3 As for Lane 2.

Lane 4 SDS extract of *M. hyopneumoniae* strain Beaufort.

Lane 5 SDS extract of *M. hyopneumoniae* strain 10110.

FIGURE 3: Western blots of Triton X-114 extracted antigens from *M. hyopneumoniae* strain Beaufort, probed with serum and supernatant antibody probes.

Lane 1 No antibody control.

Lane 2 Dookie pig serum control 1/200.

Lane 3 Pig 105 supernatant.

Lane 4 Pig 1 supernatant.

Lane 5 Dookie pig supernatant.

FIGURE 4: Western blots of SDS extracted antigens from *M. hyopneumoniae* strain Beaufort probed with paired serum and supernatantantibody probes. Fractionation of antigens on SDS Polyacrylamide gel (12.5%).

Lane 1 a) Pig 453 supernatant.

b) Pig 453 serum 1/100.

Lane 2 a) Pig 105 supernatant.

b) Pig 105 serum 1/100.

Lane 3	a) Pig 1 superanatant.
	b) Pig 1 serum 1/100.
Lane 4	a) Pig 15 supemanant.
	b) Pig 15 serum 1/100.
Lane 5	a) Dookie supematant.
	b) Dookie serum 1/100.
Lane 6	No antibody control.

FIGURE 5: Western blots of SDS extracted antigens from *M. hyopneumoniae* strain Beaufort probed with paired serum and supematantantibody probes. Fractionation of antigens on SDS Polyacrylamide gel (10.0 %).

Lane 1	a) Pig 453 supernatant.
	b) Pig 453 serum 1/100.
Lane 2	a) Pig 105 supematant.
	b) Pig 105 serum 1/100.
Lane 3	a) Pig 1 supematant.
	b) Pig 1 serum 1/100.
Lane 4	a) Pig 15 supematant.
	b) Pig 15 serum 1/100.
Lane 5	a) Dookie supernatant.
	b) Dookie serum 1/100.
Lane 6	No antibody control.

B FIGURE 6: The entire 48 k gene sequence (SEQ ID NO:1).

B FIGURE 7: tHE 48kDa protein sequence of the 48k gene sequence (SEQ ID NO:2).

EXAMPLE 1

Mycoplasma hyopneumoniae media

Friss Media

Hovind-Hougen, K., Friss, N.F., Research in Veterinary Science, 1991, 51, pp 155-163, "Morphological & Ultrastructural Studies of M flocculare and M hyopneumoniae in vitro".

250 ml Hanks BSS
140 ml Water
1.5 gm Brain Heart infusion
1.6 gm PPLO Broth w/o CV
Autoclave at 120°C for 20 minutes
18 ml Yeast Extract (100g YSC-2 Sigma in 750 ml)
3.7 ml 0.2% DNA in 0.1% Na_2CL_3
5.14 ml 1% -NAD
0.6 ml 1% Phenol red

Adjust to pH 7.3 to 7.4
Filter through 0.45 um, 0.2 um membrane, store at 4°C.
Add sterile Horse or Pig serum to 20%
and Antibiotics prior to use

Etheridge Media

Etheridge, J.R., Cottew, G.S., Lloyd, L.C., Australian Veterinary Journal, 1979, August 55, pp 356-359, "Isolation of Mycoplasma hyopneumoniae from lesions in experimentally infected pigs".

Materials	For 600 mls
Hanks BSS	18.9 ml
Hartleys Digest broth	1.28 gm
Heart Infusion broth	1.65 gm
Lactalbumin hydrolysate	2.21 gm
Glucose	4.41 gm
Yeast Extract autolysate	8.82 ml
Pig Serum (filtered)	163 ml
1% NAD	6.17 ml
1% Phenol red	1.32 ml
0.2% DNA in 0.1% Na_2CO_3	4.41 ml

Make up to 600 ml with MQ water (about 350 - 400 ml)

Adjust pH to 7.4 and filter through: 3.0 um, 0.8 um, 0.45 um, 0.2 um.

Store at 4°C.

Development of Immune Sows

Cull sows and naive gilt (unmated sow designated Dookie).

Challenged on numerous occasions, with culture grown M. hyopneumoniae and lung homogenate. Given intranasally and intratracheally. Period of challenge - from September, 1991 to 21st January, 1992.

Tiamulin antibiotic given 31st January, 1992 to 4th February, 1992. Rested for approximately 8 weeks.

Infectious Challenge

120 ml of frozen culture of M. hyopneumoniae strain Beaufort, spun down (12,000 xg, 20 min.) and resuspended in 50 ml complete medium and cultured overnight at 37°C. The overnight culture was centrifuged (12,000 xg, 20 min.) and the Mycoplasma cells resuspended in 10 ml serum free Mycoplasma culture medium. The 10 ml of concentrated mycoplasma was administered to anaesthetised immune sows via a catheter to ensure the inoculum was placed into the trachea.

Three of four days post-challenge, the sows were killed, and lymph nodes draining the lungs taken - these included the left and right tracheobronchial lymph nodes, and the lymph nodes located at the bifurcation of the trachea.

Antibody probes were prepared from pig lymph nodes and utilised to detect putative protection antigens as described in Australian Patent Application 49035/90 referred to above. Separate cell cultures were obtained from individual lymph nodes. Culture supernatants were harvested after 5 days of culture.

Antigen Preparation

Mycoplasma hyopneumoniae strain Beaufort was cultured in Etheridge media until the pH had dropped to between 6.8 and 7.0. Cells of M. hyopneumoniae were harvested from culture by centrifugation at 12,000 xg for 20 min., washed 4 times with either sterile PBS or 0.25 M NaCl and then the pelleted cells extracted with one of the following.

(i) Sodium dodecyl sulphate (SDS)

The cell pellet was resuspended in 0.2% SDS and extracted for 2 hours at 37°C. Insoluble material was pelleted from the extract at 12,000 xg for 10 min. and the soluble extract run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

(ii) Triton X-114

The method of Bordier (J. Bio. Chem. 1981, 256:1604-1606) was used to selectively extract membrane proteins using the non-ionic detergent Triton X-114.

The cell pellet was resuspended in cold PBS to 2 mg/ml protein and a cold pre-condensed solution of TX-114 added to give a final concentration of 1% (v/v) TX-114. Extraction was achieved by incubation overnight at 4°C with gentle mixing. Insoluble material was removed by centrifugation at 12,000 xg for 20 min. at 4°C. The Triton X-114 soluble membrane proteins were then obtained by achieving a phase separation at 37°C.

Proteins soluble in TX-114 phase were precipitated with 80% ethanol in the presence of carrier dextran (80,000 molecular weight) at -70°C overnight. The proteins were collected by centrifugation at 12,000 xg for 30 min. and dissolved to 500 ug/ml in 4 M urea.

Identification of Antigens

Six antigens were identified utilising the above- mentioned technique. The identified antigens were those that were consistently identified by the antibody probes from the immune cultures and the Dookie gilt. The results are summarised in Table 1.

TABLE 1

Molecular Weight (kD)	Characteristics
110-114	SDS Extracted
90-94	SDS Extracted
72-75	Triton X-114 Extracted
60-64**	SDS Extracted. Partitions to aqueous phase of Triton X-114 extract.
52-54	Triton X-114 Extracted
46-48	Triton X-114 Extracted

** Two antigens of approximate molecular weight 62 kD were identified.

Molecular Weight (kD)	Amino Acid Sequence
46-48	48 K N-Terminal: (SEQ ID NO:3) AGXGQTESGSTSDSKPQAETLKHKV
	48 K CNBR F 1: (SEQ ID NO:4) TIYKPDKVLGKVAVEVLRVLIAKKNKASR
	48 K CNBR F 2: (SEQ ID NO:5) AEQAITKLKLEGFDTQ
	48 K CNBR F 3: (SEQ ID NO:6) KNSQNKIIDLSPEG
52-54	52 K N-Terminal: (SEQ ID NO:7) AGXWAKETTKEEKS
	52 K CNBR F 1: (SEQ ID NO:8) AWVTADGTVN
	52 K CNBR F 2: (SEQ ID NO:9) AIVTADGTVNDNKPNQWVRKY
60-64	62 K N-Terminal: (SEQ ID NO:10) MKLAKLLKGFX (N/L)(M/V) IK
60-64	62 K N-Terminal (SEQ ID NO:11) ADP(F/I)(R/E)Y(V/A)PQG(Q/A)X(M/N)VG

B	72-75	(SEQ ID NO: 12) 74 K N-Terminal: AGXLQKNSLLEEVWYLAL
B		(SEQ ID NO: 13) 74 K CNBR F 1: AKNFDFAPSIQGYKKIAHEL
B		(SEQ ID NO: 14) 74 K CNBR F 2: NLKPEQILQLLG
B		(SEQ ID NO: 15) 74 K CNBR F 3: LLKAEXNKXIEEINTXLDN

CNBR - Cyanogen Bromide fragment

X denotes an undetermined amino acid

(A/B) - residue may be A or B

PCR of 48kDa Gene

Polymerase Chain Reaction (PCR) oligonucleotide primers were designed from the amino acid sequences obtained from the N-terminal and internal cyanogen bromide (CNBr) derived peptides. Inosine (I) was substituted at positions of high redundancy. The following primers were used in a standard PCR assay, run on a Bartelt Gene Machine Robotic thermal cycling instrument.

B (SEQ ID NO: 16)
Oligo 48 K CNBr F 1: ACIAACGACGAGAAGCCICAGGC
T T A A A

B (SEQ ID NO: 17)
Oligo 48 K CNBr F 2: TTIAGCTTIGTGATIGCCTGCTC
AT A T T
T

B (SEQ ID NO: 18)
Oligo 48 K CNBr F 3: AGGTCGATGATCTTCCAICC
AA A A T T
T T

The resulting PCR products were visualised on a 1.5% agarose gel, excised, and purified using Prep-a-Gene (BioRad). They were cloned by standard techniques into a dideoxy tailed T-vector (Holton and Graham, Nucleic Acids Research 19: 1155, 1991) and the nucleic acid sequence determined. The PCR product, obtained from the reaction using primers F1 and F2 shown above, was of

approximately 810 base pairs and was shown by sequencing to code for the previously determined amino acid sequence of the purified native 46-48kDa protein.

Genomic clone isolation at 48 k gene

The entire 48k gene and 48kDa protein (Figures 6 and 7) has been isolated and sequenced. The gene was obtained from an *M. hyopneumoniae* genomic library made by digesting genomic DNA with the restriction enzyme CLA I and ligating the fragments into the vector pBluescript (Stratagene). The ligated product was then electroporated into *Escherichia coli* strain SURE (Stratagene) and the cells plated on Luria Broth agar plates containing 100 µg/ml Ampicillin (LB-Amp). The library was screened by DNA hybridisation with a polymerase chain reaction (PCR) product specific for the 48 kDa protein. Positive clones were grown in LB-Amp, the cells harvested and the DNA isolated and partially sequenced for confirmation.

The positive clone pC1-2 was entirely sequenced and the protein sequence deduced. This was compared to the protein sequence obtained from the N terminus and Cyanogen Bromide fragments of the 48 kDa protein to show the that the gene encoded the desired protein.

Adjuvant Selection

Young piglets, 5-7 weeks of age, were immunised with identified antigen(s). The antigens include Triton X-114 extract and identified proteins of 46-48, 52-53, 60-64, 70-75, 90-94 and 110-114 kD, either singly or in combination. An immunising dose of antigen, containing between 5-100 µg protein, was given by intramuscular injection in combination with an adjuvant. An adjuvant is selected from:

(i) Seppic Montanide ISA-50
(ii) Quill A and other derivatives of saponin.
(iii) oil in water emulsion employing a mineral oil such as Bayol F/Arlacel A.
(iv) oil in water emulsion employing a vegetable oil such as corn oil.

safflower oil or other with lecithin as emulsifier,

(v) aluminium hydroxide gel, and

(vi) nonionic block polymer such as Pluronic F-127 produced by BASF (U.S.A.).

Immunising doses were given at 2-4 week intervals, the number of doses being dependent on the adjuvant and amount of antigen, but preferably 2 to 3 doses are given.

Adjuvants were treated on the basis of being able to induce antibody titres, as measured by ELISA, and by assessment of induced cell-mediated immunity as tested by Delayed-Type Hypersensitivity (DTH) reaction.

The results clearly show that mineral-oil type adjuvants are consistently superior at inducing antibody titres and DTH responses (Table 2). In particular an adjuvant marketed under trade designation Montanide ISA-50 and available from Seppic, Paris, France has been found to be suitable.

TABLE 2

GROUP	Animal Number	DTH 24 Hour Response	DTH 48 Hour Response	Antibody Levels (450 nm)
	19	0	0	0.061
	11	0	0	0.010
CONTROL	1	-	-	0.005
(Unvaccinated)	15	0	0	0.038
	7	0	0	0.005
	18	+	0	0.753
QUIL A	25	+	0	0.788
	17	0	0	0.638
	168	-	±	0.642
	169	+++	0	0.316
	22	0	0	0.621
VEG. OIL	4	+	0	0.666
	6	+	+	0.239
	13	+++	++	0.457
	14	+++	++	1.086
	5	+++	++	1.024
MIN. OIL	23	+++	+	0.864
	16	+++	0	0.975
	21	+	±	0.954

TABLE 2: Antibody levels and DTH responses in pigs measured 2 weeks after the third injection of antigen from *M. hyopneumoniae*. (- = no response; ± = faint reddening; + = faint reddening and swelling; ++ = reddening; +++ = swelling with or without reddening).

Protection Pen Trial

Groups of 9 young piglets, 6 weeks of age, were immunised with purified and semi-purified antigens as shown in Table 3 below. The antigens were purified on reversed-phase HPLC using a formic acid solvent system with an acetonitrile gradient.

Antigens were resolubilised in 4 Molar urea before incorporation in mineral oil adjuvant.

The immunisation schedule is as shown in Table 2.

TABLE 3

Protocol for Pen Trial of Antig ns of Mycoplasma Hyopneumoniae

VACCINATIONS & BLEEDS

Treatment	Day Number
1st Vaccination	0
2nd Vaccination	14
3rd Vaccination	50
Infectious Challenge	64
Slaughter	91

ANTIGEN DOSES

Partly Purified	1st & 2nd Vaccns. 50μg COMPLEX ANTIGEN/DOSE
62 kD	3rd Vaccn. - 220μg PARTIALLY PURIFIED ANTIGEN/DOSE
(Purified)74+52kD	1st Vaccn. 20μg total protein/DOSE
	2nd Vaccn. 13μg total protein/DOSE
	3rd Vaccn. 17μg total protein/DOSE
(Purified) 48KD	1st Vaccn. 20μg/DOSE
	2nd Vaccn. 18μg/DOSE
	3rd Vaccn. 27μg/DOSE

ALL PROTEIN ESTIMATIONS DONE BY "BCA" PROTEIN ASSAY (Pierce, Illinois, U.S.A.

Protection from infection with Mycoplasma hyopneumoniae was assessed by infectious challenge 2 weeks after the final immunisation. Infectious challenge was achieved by intranasal administration of 10ml of a 10% (w/v) lung homogenate, prepared from infected lung, and by housing test piglets with

previously infected piglets. Four weeks after infectious challenge, the animals were killed and the extent and degree of lung lesions assessed (Table 4).

TABLE 4

Pen Trial of Antigens of Mycoplasma Hyopneumoniae

Group No.	No. Pneumonia Free (%)	Median Lung Lesion Score	% Reduction (from Median)
Controls	1 (11)	13	0%
62 kD	0 (0)	5	61%
74+52 kD	3 (33)	6.75	48%
48 kD	2 (22)	6.25	52%

REFERENCE

Warren H.S. and Chedid, L.A., Future Prospects for Vaccine Adjuvants CRC Critical Reviews in Immunology 8 : 83-108, 1988.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

add B'